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SKI Activates Wnt/β-Catenin Signaling in Human Melanoma[1]

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ABSTRACT

Overexpression of the oncoprotein SKI correlates with the progression of human melanoma *in vivo*. SKI is known to curtail the growth inhibitory activity of tumor growth factor β through the formation of repressive transcriptional complexes with Smad2 and Smad3 at the $p21^{Waf-1}$ promoter. Here, we show that SKI also stimulates growth by activating the Wnt signaling pathway. From a yeast two-hybrid screen and immunoprecipitation studies, we identified the protein FHL2/DRAL as a novel SKI binding partner. FHL2, a LIM-only protein, binds β-catenin and can function as either a transcriptional repressor or activator of the Wnt signaling pathway. SKI enhanced the activation of *FHL2* and/or *β-catenin*-regulated gene promoters in melanoma cells. Among the SKI targets were microphthalmia-associated transcription factor and Nr-CAM, two proteins associated with melanoma cell survival, growth, motility, and transformation. Transient overexpression of SKI and FHL2 in $ski^{-/-}$ melanocytes synergistically enhanced cell growth, and stable overexpression of SKI in a poorly clonogenic human melanoma cell line was sufficient to stimulate rapid proliferation, decreasing the number of cells in the G_1 phase of the cell cycle, and dramatically increasing clonogenicity, colony size and motility. Taken together, these results suggest that by targeting members of the tumor growth factor β and β-catenin pathways, SKI regulates crucial events required for melanoma growth, survival, and invasion.

INTRODUCTION

The incidence of malignant melanoma is increasing steadily and is a leading cause of death in the United States (1). The oncogene *SKI* was recently reassigned to chromosome 1p36 (2, 3), a common region of alterations in human cancers, including melanomas (4). The levels of SKI expression correlate with the malignant grade of human melanoma (5). In preinvasive melanomas *in situ*, SKI is expressed predominantly in the nucleus of intraepidermal melanoma cells. However, in primary invasive and metastatic melanomas, SKI localizes to both the nucleus and cytoplasm. Overexpression of SKI in melanomas results from yet-to-be-defined transcriptional and/or posttranscriptional events, as Southern blot analysis did not detect alterations in restriction enzyme patterns or an increase in gene dosage (6).

Human *SKI* is the homologue of *v-ski* (7), the transforming gene of the defective Sloan-Kettering virus (8). High levels of SKI curtail the TGF-β[4] response by forming repressor complexes with the Smad proteins, which specifically bind Smad binding elements (SBE) (9–12). Binding of SKI to Smad2/Smad3 causes the histone acetyltransferase p300 to dissociate from the Smad2/3 complex and promotes the association of mSin3A and histone deacetylases (11). The Smad proteins regulate transcription of the cyclin-dependent kinase inhibitor $p21^{Waf-1}$ (13), which together with $p15^{INK4b}$ (14), mediate the antiproliferative and tumor suppressor activity of TGF-β. Thus, high levels of SKI prevent $p21^{Waf-1}$ induction through a Smad-dependent mechanism that involves transcriptional repression (5). Importantly, down-regulation of SKI protein levels by antisense *SKI* vectors restores TGF-β-mediated growth inhibition in melanomas (5). Therefore, high SKI levels are necessary and sufficient to block the inhibitory activity of TGF-β. In addition, SKI, in association with the SKI-interacting protein Skip, suppresses the transcriptional repressor activity of the retinoblastoma (pRB) tumor suppressor (15). Ski can also alter cellular differentiation by interacting with the retinoic acid receptor complex and repressing transcription from a retinoic acid response element (16). Thus, SKI represses powerful mechanisms that suppress cellular proliferation and alter differentiation programs, suggesting that overexpression of SKI results in at least two of the essential alterations that dictate malignant growth.

The Wnt signaling pathway controls cell fate determination in neural crest cells, which give rise to melanocytes (17). Activation of Wnt signaling involves the inhibition of β-catenin degradation by the proteasome, which results in its nuclear accumulation and transcriptional activation of LEF/TCF target genes (reviewed in Refs. 18, 19). Transfection of β-catenin to mouse neural crest results in the expansion of the melanocyte lineage (20). β-catenin/LEF activates the gene coding for MITF (21), which controls the commitment, proliferation, and survival of the melanocyte lineage (22).

Malignant transformation is associated with major changes in the organization of the cytoskeleton, decreased adhesion, and aberrant adhesion-mediated signaling (reviewed by Refs. 18, 22). An additional β-catenin target gene is *Nr-CAM* (23), a neural cell adhesion molecule (24), which is overexpressed in human malignant melanomas but not normal melanocytes, and its overexpression in cells enhances motility and transformation and produces rapidly growing tumors in mice (23).

β-Catenin interacts with several transcriptional coregulators, including p300/CBP (25), BRG-1 (26), and the LIM-only protein FHL2 (27). LIM domains are characterized by the cysteine-rich consensus $CX2CX_{16\text{-}23}HX_2CX_2CX_2CX_{16\text{-}21}CX_{2\text{-}3}$(C/H/D) (28) and function as adapters and modifiers in protein interactions (29). FHL2 is particularly intriguing because it can function as a repressor or activator of β-catenin in a cell type-dependent fashion (27). FHL2 also interacts with other proteins; it functions as a tissue-specific coactivator of the androgen receptor (30), it coregulates the transcription factors cAMP-responsive element binding protein and CREM (31), and it corepresses the promyelocytic leukemia zinc finger protein (32). FHL2 transmits Rho signals from the cell membrane into the nucleus (33) and binds to the cytoplasmic domains of several α and β integrin chains (34).

Here, we demonstrate that SKI interacts with FHL2 and potentiates

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[4] The abbreviations used are: TGF-β, tumor growth factor β; MITF, microphthalmia-associated transcription factor; CHO, Chinese hamster ovary; DCT, Dopachrome tautomerase; FBS, fetal bovine serum; β-gal, β-galactosidase; DBD, DNA binding domain; Ab, antibody; RT-PCR, reverse transcription-PCR.

the function of β-catenin in a melanoma-specific manner, inducing MITF and Nr-CAM, proliferation, cell cycle alterations, and features of melanoma progression.

MATERIALS AND METHODS

Cell Culture, Proliferation, and Migration Assays

Cell Culture

The human melanoma cell lines UCD-Mel-N, derivative lines expressing the human *ski* gene (UCD(SKI^+) (12) or the human *ski* plus *fhl2* genes (UCD(SKI^+/$FHL2^+$), and A375 human melanoma cells were cultured in DMEM/F-12 medium (Life Technologies, Inc., Grand Island, NY) containing 8% FBS, 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 0.5 μg/ml transferin (Sigma-Aldrich), 5 ng/ml epidermal growth factor (Sigma-Aldrich), and 1% penicillin/streptomycin (Life Technologies, Inc.; Ref. 12). Both cell lines, UCD-Mel-N and A-375, express identical endogenous levels of the SKI protein (data not shown). The (UCD(SKI^+) cell line was generated by infection of the parental cell line with recombinant retroviruses carrying the human *SKI* gene (pBabe-SKI), whereas the UCD(SKI^+/$FHL2^+$) was generated by transfecting UCD(SKI^+) cells with an HA-FHL2-expression plasmid [pcDNA 3.1(+)/HA-FHL2] carrying a hygromycin-resistant marker. 293T cells and HeLa cells were grown in DMEM (high glucose; Life Technologies, Inc.) containing 10% FBS and 1% penicillin/streptomycin, whereas CHO cells were cultured in F-12 medium, 10% FCS, and 1% penicillin/streptomycin. Primary mouse melanocytes cultures were established from E18.5 fetuses. Skins were incubated in 0.25% trypsin solution in Ca^{2+}- and Mg^{2+}-free HBSS at 37°C for 2–3 h to allow separation of the epidermis from the dermis. The dermal layer was placed in a 15-ml centrifuge tube containing culture medium and vigorously vortexed for 1–2 min to obtain a cell suspension containing dermal melanocytes. The mouse melanocyte growth medium consists of F-12 medium supplemented with 8% FCS, 1 μl/ml pituitary extract (BioWhittaker, Baltimore, MD), 45 μg/ml 12-*O*-tetradecanoylphorbol-13-acetate (Sigma-Aldrich), 0.1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), and 1% penicillin/streptomycin. Genotyping was used to define melanocyte cultures derived from homozygous mutant $ski^{-/-}$ mice (35).

Cell Growth Assays

Transiently Transfected Cells. The $ski^{-/-}$ mouse melanocytes were transfected with SKI, FHL2, or a combination of SKI plus FHL2 plasmids together with a cytomegalovirus-β-gal expression vector at a ratio of 10:1. Cells were stained for β-gal activity at day 3 after transfections. Cell growth was calculated by counting the number of blue-stained cells in each colony. At least 300 cells were counted/60-mm dish.

Stably Transfected Cells. We used the Cell Proliferation Kit II (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt) according to the instructions provided by the manufacturer (Roche, Indianapolis, IN). The cell labeling mixture provided by the kit was added 3 h (day 0), 51 h (day 2), and 99 h (day 4) after seeding the human melanoma cells to the tissue culture plates. After incubation at 37°C for 4 h, the medium was aspirated and absorbance determined at 492 and 690 nm, respectively.

Motility Assays. Confluent UCD and UCD(SKI^+) melanoma cell dishes were scraped with the tip of a micropipette, and the culture medium was replaced with fresh medium. Photographs were taken at 0 and 24 h after wounding the monolayer.

Plasmids

pSG5myc-SKI and pSG5HA-FHL2 plasmids were constructed by inserting PCR amplification products from human SKI cDNA and FHL2cDNA into *Bgl*II and *Xho*I sites in the pSG5myc or pSG5HA vectors. The SKI deletion mutants myc-SKI (1-261), myc-SKI (1-492), myc-SKI (Δ46-260), myc-SKI (Δ46-260 and Δ493-728), myc-SKI (99-272), myc-SKI (197-330), and myc-SKI (556-728) were constructed by cloning the corresponding PCR amplified fragments into the *Bgl*II and *Xho*I sites of pSG5-myc. The FHL2 deletion mutants HA-FHL2 (1-95), HA-FHL2 (1-162), HA-FHL2 (1-220), HA-FHL2 (100-220), HA-FHL2 (100-279), and HA-FHL2 (162-279) were obtained by cloning the respective PCR fragments into the *Bgl*II and *Xho*I sites of pSG5-HA. All vectors were sequenced to confirm appropriate reading frames. PGL3OT/OF and β-catenin plasmids were kindly provided by Bert Vogelstein and Andreas Hecht, respectively. The human MITF promoter construct (pGL2-hMip) (−387-to +97) and a deletion construct (−175) were described previously (36, 37). The Nr-CAM-pA3-Lux reporter plasmid was described previously (23).

The Yeast Two-Hybrid Screen

The yeast-two hybrid screen using SKI and Gal4 vectors was performed as described previously (12). Briefly, colonies that grew in the presence of 3-aminotriazole (10 mM) and in the absence of adenine and histidine were additionally analyzed for β-gal activity by a liquid assay. cDNA inserts derived from triple positive (adenine, histidine, and LacZ) yeast colonies were tested for bait specificity by retransformation with different Gal4-DBD fusion proteins and the pAS2–1 vector expressing the Gal4-DBD only.

Immunoprecipitation

293T cells were transfected with FuGENE6 (Boehringer Mannheim) according to the manufacturer's instructions, solubilized in 0.5% NP40, 50 mM HEPES (pH 7.9), 2 mM EDTA, and 100 mM NaCl containing a protease inhibitor mixture (Boehringer Mannheim) and centrifuged at 10,000 × *g* for 30 min at 4°C. Supernatants were incubated for 1 h at 4°C with 50 μl of immobilized protein G-agarose beads (Pierce) followed by centrifugation. The resulting supernatants were incubated with mouse anti-myc, mouse anti-HA (both Abs from Boehringer Mannheim) or normal mouse serum for 2 h at 4°C. Immunocomplexes collected by centrifugation after incubation with protein G-agarose beads were analyzed by SDS-PAGE and immunoblotting with either anti-myc or anti-HA Abs using the enhanced chemiluminescence detection system (Amersham-Pharmacia). Protein extracts from UCD(SKI^+/$FHL2^+$) cells were immunoprecipitated with an anti-HA Ab and immunoblotted with an anti-SKI Ab (12).

Transfections and Luciferase Activity Assays. 293T, HeLa, CHO, and the melanoma cell lines UCD-Mel-N and A375 were transfected using FuGENE 6 reagent, as specified by the manufacturer (Boehringer Mannheim). Transfections used 0.3 μg each of GL3-OT-Lux, pGL3-OF-Lux, TCF-Lux, MITF-pGL3-Lux (−387 to +97), MITF-pGL3-Lux (−179 to +97), and Nr-CAM-pA3-Lux reporter plasmids and combinations of 0.6 μg each of β-catenin, SKI, FHL2, and p300 plasmids together with 20 ng of a β-gal expression plasmid for normalization of transfection efficiencies. Empty vectors and experimental conditions have been balanced for DNA content. Each transfection was carried out in triplicate. Luciferase and β-gal activity were measured 48 h after transfection using the Luciferase Assay System kit and β-gal Assay kit (Promega).

RT-PCR. UCD melanoma cells expressing the human *ski* gene (UCD(SKI^+) or an empty vector were used to determine mRNA levels of SKI, MITF, and Nr-CAM by RT-PCR analysis. Total RNA was purified using the TRIzol reagent (Invitrogen). Ten μg of total RNA were used for reverse transcription using oligo(dT) primers (Stratagene). PCR was performed using the following primers: Nr-CAM, 5′-ACACTCTACCAGGTCATTGC-3′ and 5′-TAGATGATCCTTGTCAACAG-3′; MITF, 5′-GATGACATCATTAGCCTAGA-3′ and 5′-GCGCATGTCTGGATCATTTG-3′; SKI, 5′-CCCTGCAAGAAGGAGCTGGCC-3′ and 5′-TCCTCTGAGCCTCCGGCCTCC-3′; and glyceraldehyde-3-phosphate dehydrogenase, 5′-TCAGTGGTGGACCTGACCTG-3′ and 5′-TCGTTGTGATACCAGGAAAT-3′. The conditions for PCR were 94°C (3 min) for 1 cycle, 94°C (45 s), 56°C (1 min), and 72°C (2 min) for 28 cycles. PCR products were electrophoresed on agarose gels and photographed.

RESULTS

Identification of FHL2 as a SKI Binding Protein. Using a yeast two-hybrid human melanoma library and full-length human *ski* as bait, we previously demonstrated that SKI interacts with and represses the activity of Smad2 and Smad3, two proteins involved in the TGF-β signaling pathway (12). Additional screening identified a third open reading frame that encodes a 279 amino acid protein with a predicted molecular weight of M_r 32,000. A human genome database search

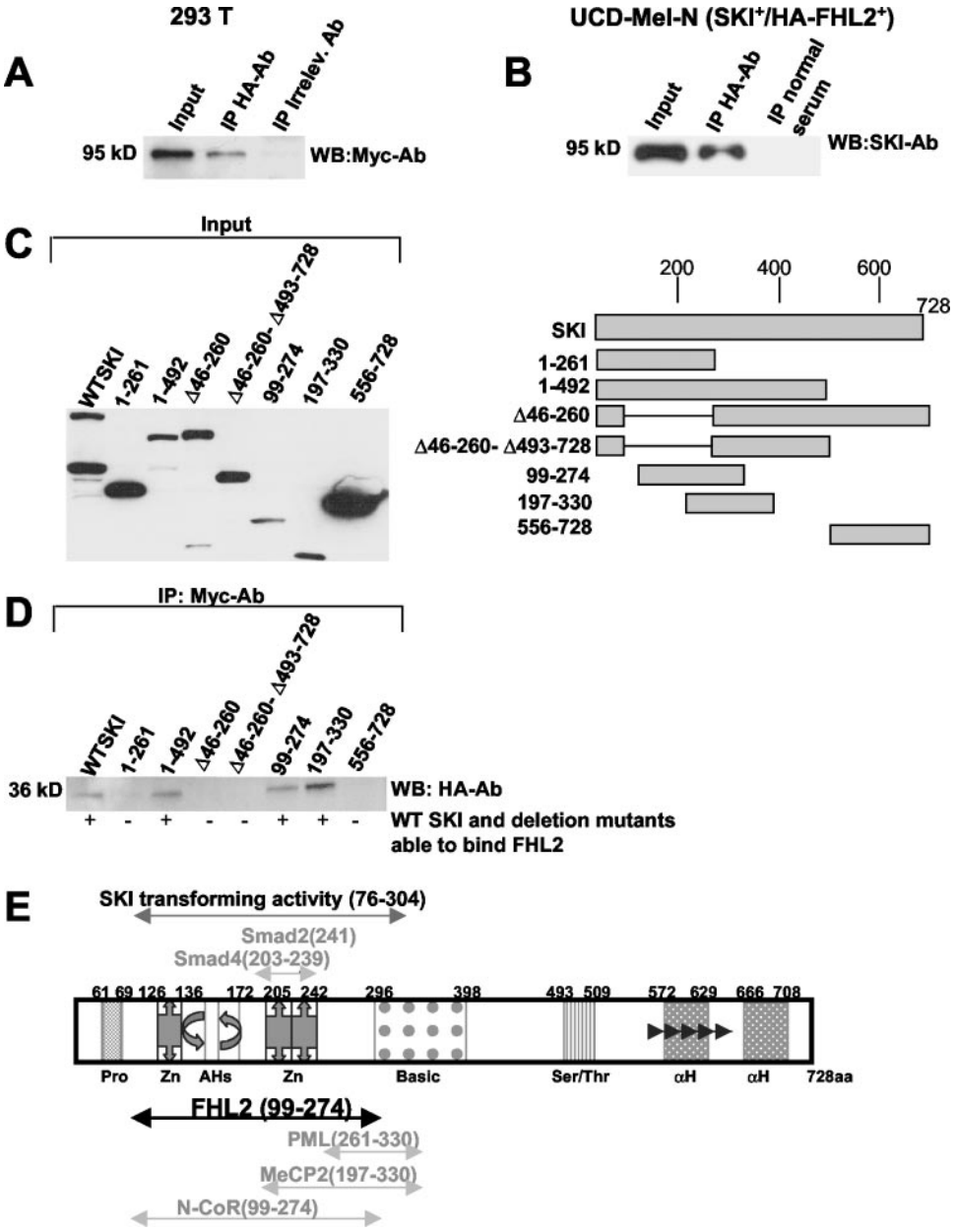

Fig. 1. SKI domains required for association to FHL2. *A*, lysates of 293T cells cotransfected with Myc-SKI and HA-FHL2 expression plasmids were analyzed by immunoprecipitation (IP) with anti-HA Ab, followed by Western blotting with myc Ab. *B*, lysates of the human melanoma cells UCD(SKI$^+$/HA-FHL2$^+$), stably expressing SKI plus HA- FHL2, were analyzed by co-IP with an anti-HA Ab, followed by Western blotting with a SKI Ab. *C*, 293T cells were cotransfected with Myc-tagged full-length SKI or SKI deletion mutants. The blot shows the expression of myc-tagged SKI deletion mutants. *D*, the interaction domain of SKI with FHL2 was analyzed by coimmunoprecipitation with Myc-tagged Abs, followed by Western blotting with an anti-HA Ab. The blot shows results from coimmunoprecipitations. *E*, a diagram of the SKI protein and interaction domains with several protein partners. Symbols indicate: AH, helix-loop-helix motif; αH, a unique tandem repeats of α helical domains that may be involved in the dimerization of the SKI family through coiled-coil interactions; basic region, region with high content of basic amino acids; Pro, proline-rich region; Ser/Thr, putative serine/threonine phosphorylation sites; Zn, putative zinc fingers; *arrowheads* indicate three tandem repeats of 25 amino acids located at residues 572–645. Domains required for binding to SKI protein partners are indicated in the figure.

revealed that this protein is identical to FHL2/DRAL, a four and a half LIM domain protein (38–40). Retransformation assays showed that the FHL2-GAL4-DBD fusion protein interacts specifically with the SKI bait because no interaction was detected with either GAL4-DBD alone or GAL4-DBD-p53 (data not shown). To confirm the yeast two-hybrid results, we examined the interaction between SKI and FHL2 in cells. 293T cells were cotransfected with vectors that expressed Myc-tagged SKI and HA-tagged FHL2 (Fig. 1*A*). Coimmunoprecipitation results indicated that the SKI/FHL2 interaction also occurred in cells (Fig. 1*A*). Thus, the anti-HA Ab coprecipitated myc-SKI, whereas control serum did not. To confirm these results in stably transfected cells, we generated the human melanoma cell line UCD(SKI$^+$/FHL2$^+$) that stably overexpressed SKI plus HA-tagged FHL2 (described in "Materials and Methods"). Immunoprecipitations with an anti-HA-Ab demonstrated HA-FHL2/SKI complex formation (Fig. 1*B*).

To identify regions of the SKI protein required for binding to FHL2, we performed coimmunoprecipitations using several SKI deletion mutant proteins (Fig. 1, *B* and *C*). The results indicated that a minimal domain that includes amino acids 260–274 in SKI mediated the interaction, but additional amino acids that reside within the 99–274 domain were also required for association with FHL2 (Fig. 1*D*, *bottom panel*). This region overlaps with the binding of several other proteins, including N-CoR, MeCP2, PML, Smad2, and Smad4 (Fig. 1*E*).

To identify the region in FHL2 required for binding SKI, we performed coimmunoprecipitations using several FHL2 deletion mutant proteins (Fig. 2*A*). The results indicated that amino acids 1–95, containing one and a half LIM domains of FHL2, interact with SKI (Fig. 2*A*). The SKI binding domain partially overlaps with the β-catenin binding site (Fig. 2*B*; Ref. 27).

SKI Is a Potent Stimulator of Wnt/β-Catenin Signaling in Human Melanoma Cells. FHL2 can associate with β-catenin and functions as an activator or repressor of transcription in the Wnt signaling pathway, depending on the cellular context (27). Those studies demonstrated that transfection of FHL2 and a stable β-catenin

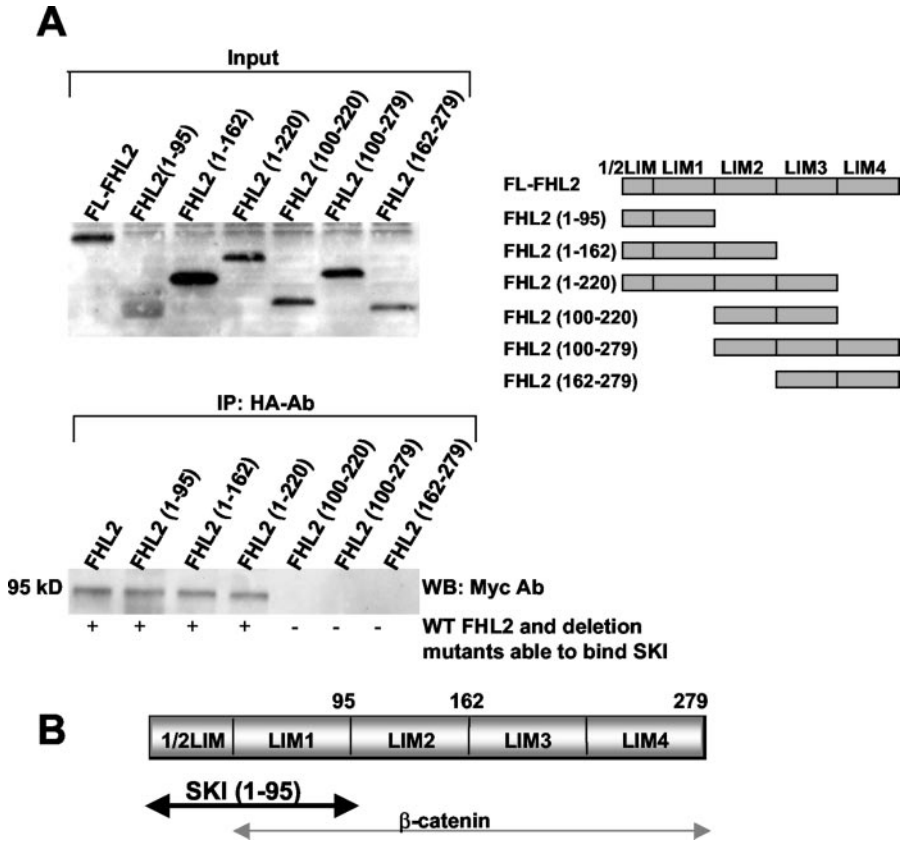

Fig. 2. FHL2 domains required for association with SKI. *A*, 293T cells were cotransfected with full-length (FL) Myc-tagged SKI and HA-tagged, FL-FHL2, or FHL2 deletion mutants. The interaction domain of FHL2 with SKI was determined by immunoprecipitation with a HA-Ab followed by Western blotting with a Myc-Ab. The *top blot* shows the expression of HA-tagged FHL2 constructs. The blot in the *bottom panel* shows immunoprecipitation results followed by Western blotting with an anti-Myc Ab. *B*, a diagram of FHL2 indicating domains required for SKI and β-catenin binding.

(S33A) mutant activated a β-catenin-regulatable synthetic promoter (Topflash) in human kidney (293T) cells. To determine whether SKI alters the regulation of β-catenin-responsive promoters by FHL2, we cotransfected 293T cells with pGL3-OT, an improved version of the Topflash vector (41), together with SKI, FHL2, and β-catenin expression plasmids. The β-catenin coactivator p300/CBP (25) was used as a positive control. As expected, β-catenin plus FHL2 stimulated more the pGL3-OT promoter than β-catenin alone (Fig. 3*A*, compare *Lane 7* with *Lane 3*). Surprisingly, SKI plus β-catenin stimulated the activity of this promoter to a similar level (Fig. 3*A*, *Lane 8*), whereas SKI alone did not activate this promoter significantly (Fig. 3*A*, *Lane 5*). Importantly, SKI in combination with FHL2 and β-catenin showed a potent transactivation activity, which was additionally stimulated by p300/CBP (Fig. 3*A*, compare *Lane 12* with *Lane 10*). SKI activity on the pGL3-OT promoter was specific because it was almost completely abolished when the LEF-1-binding sites that are required for β-catenin-mediated activation together with LEF/TCF factors (42) were mutated (pGL3-OF; Fig. 3*B*).

Deregulation of β-catenin signaling is associated with the genesis of a number of human malignancies, including melanoma (43). However, although β-catenin mutations are rare in primary malignant melanoma, its nuclear and/or cytoplasmic accumulation is frequently observed in melanoma (44). Because β-catenin signaling can also regulate the proliferation of B16 mouse melanoma cells (37), we sought to determine whether SKI and FHL2 could affect β-catenin signaling in human melanomas. When SKI, FHL2, and β-catenin were cotransfected into the human melanoma cell lines UCD-Mel-N and A-375, the three proteins synergistically activated the pGL3OT promoter-reporter, compared with β-catenin alone (Fig. 3, *C* and *D*, compare *Lane 8* with *Lane 2*). In contrast, cotransfection of SKI, FHL2 and β-catenin slightly repressed pGL3OT promoter activity in HeLa cells (Fig. 3*E*) and completely repressed its activity in CHO cells compared with the activity of β-catenin alone (Fig. 3*F*, compare *Lane 8* with *Lane 2*). These results support the hypothesis that SKI can function as an activator or repressor of transcription, depending on the promoter and the cell type (45, 46), and in melanoma cells, SKI-containing complexes are potent inducers of the Wnt/β-catenin signaling pathway.

SKI Enhances β-Catenin-Dependent Activation of MITF and Nr-CAM Promoters in Melanoma Cells. MITF is the earliest known marker of commitment to the melanocyte lineage; once its expression is established, it is required for melanoblast survival (reviewed in Ref. 47). β-Catenin activates the MITF promoter-reporter in B16 mouse melanoma cells and modulates melanoma viability by inducing the antiapoptotic protein Bcl2 (48). To determine whether SKI is an upstream regulator of MITF, a pGL2hMIP promoter-reporter containing a 484-bp fragment of the human MITF promoter was cotransfected with SKI, FHL2, and β-catenin expression vectors into A375 and UCD-Mel-N human melanoma cells. SKI significantly enhanced the activity of the MITF promoter in the absence of exogenous FHL2 and β-catenin (Fig. 4*A*, *Lane 4*). However, additional activation was observed by cotransfection of SKI, FHL2, and β-catenin (Fig. 4*A*, *Lane 8*). It was previously shown that deletion of the LEF/TCF binding site from the MITF promoter dramatically reduced its activity in mouse melanoma cells and zebrafish (48, 49). In agreement with these observations, SKI or SKI plus β-catenin and FHL2 also demonstrated an 8–10-fold reduction in promoter activity when the TCF/LEF site was removed (Fig. 4*A*, *Lanes 2–8* and 4*B*, *Lanes 2–8*). Consistent with the results shown in Fig. 4*A*, SKI also activated the MITF promoter in the human melanoma cell line UCD (Fig. 4*C*). Importantly, SKI activity on the MITF promoter was melanoma specific because SKI repressed its activity in 293T cells (Fig. 4*D*, *Lane 4*).

Human melanoma cells expressing increased levels of Nr-CAM, a

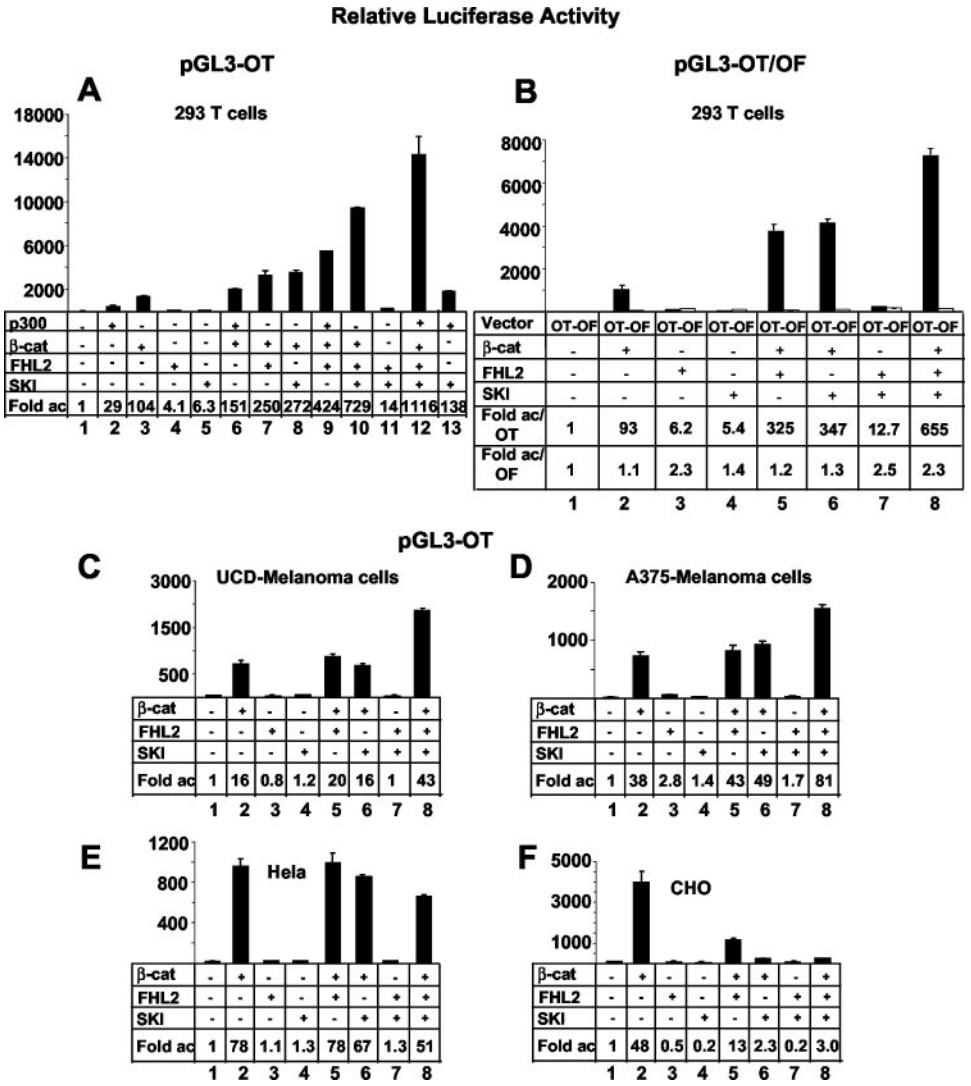

Fig. 3. SKI activates Wnt-regulated gene promoters in 293T and human melanoma cells. Different cells were transfected with various constructs and their effect on the activities of different promoter-reporters were determined. *A*, pGL3-OT-Luc activity in 293T cells. *B*, pGL3-OT and pGL3-OF-Luc activity in 293T cells. *C* and *D*, pGL3OT-Luc activity in the human melanoma cell lines UCD-Mel-N and A375. *E* and *F*, pGL3OT-Luc activity in HeLa and CHO cells, respectively. Cells were transfected with the indicated promoter-reporters in the presence or absence of SKI, FHL2, β-catenin, and p300 expression plasmids as indicated. Luciferase activity, normalized to β-gal activity, was determined as described in "Materials and Methods." The results shown are representative of at least three independent experiments. The data are the average of triplicate measurements ± the SE.

transmembrane cell adhesion protein, are highly tumorigenic in nude mice compared with cells with little or no Nr-CAM expression (23). The Nr-CAM promoter has TCF/LEF binding sites that confer β-catenin responsiveness (23). To determine whether SKI can regulate the *Nr-CAM* gene, we transfected a reporter plasmid containing 1.8 kb of the Nr-CAM promoter, SKI, FHL2, and β-catenin expression vectors into A375 and UCD melanoma cells. FHL2 alone (Fig. 4*E*, *Lane 3*) but not SKI (Fig. 4*E*, *Lane 4*) induced substantial promoter activity compared with β-catenin (Fig. 4*E*, *Lane 2*) in A375 cells. However, SKI and β-catenin synergistically activated Nr-CAM promoter activity (Fig. 4*E*, *Lane 6*), whereas no synergism was observed between β-catenin and FHL2 in the activation of *Nr-CAM* in melanoma cells (Fig. 4*E*, *Lane 5*). Intriguingly, cotransfection of SKI, FHL2, and β-catenin substantially reduced *Nr-CAM* promoter activity (Fig. 4*E*, compare *Lane 8* with *Lane 6*). In turn, SKI was a more potent activator of the *Nr-CAM* promoter (Fig. 4*F*, *Lane 4*) than β-catenin or FHL2 (Fig. 4*F*, *Lanes 2* and *3*) in UCD cells. β-Catenin and FHL2 synergistically activated the Nr-CAM promoter (Fig. 4*F*, *Lane 5*), whereas additive effects were observed between β-catenin and SKI (Fig. 4*F*, *Lane 6*). Taken together, these results suggest that SKI is a potent activator of β-catenin signaling that targets the MITF and Nr-CAM promoters in melanoma cells.

SKI Increases the Proliferation, Clonogenicity, and Motility of Human Melanoma Cells. The human SKI protein is 91% homologous to v-ski, the transforming protein of the defective Sloan-Kettering virus (8). Early studies showed that v-ski induces transformation of pigmented primary quail melanocytes (50). To assess whether SKI by itself or in association with FHL2 stimulates melanocyte proliferation and/or enhances features of melanoma progression, we used two different approaches. First, SKI, FHL2, or control vectors were cotransfected with β-gal vectors into mouse melanocytes isolated from E18.5 homozygous mutant $ski^{-/-}$ fetal skin. Dividing blue cells were observed as clusters containing two or more cells by day 3 after transfection. Transient transfection assays demonstrated that human SKI or FHL2 each induced after 3 days rapid proliferation of mouse $ski^{-/-}$ melanocytes, which was additionally enhanced by cotransfection of both plasmids (Fig. 5*A*). We have also the human melanoma cell lines UCD-Mel-N and the derivative lines stably overexpressing the human *ski* gene (UCD(SKI^+) or the human *SKI* plus *fhl2* genes (UCD(SKI^+/$FHL2^+$). Proliferation studies demonstrated that increased SKI dosage stimulated proliferation of human melanoma cells compared with empty vector controls cells, whereas overexpression of both SKI and FHL2 resulted in even greater proliferation (Fig. 5*B*). Consistent with previous publications demonstrating that overexpression of FHL2/DRAL induces apoptosis in several cell lines (51), we were unable to generate stable melanoma cells overexpressing FHL2 only because these cells formed aborted colonies containing two to four cells (data not shown). Thus, high

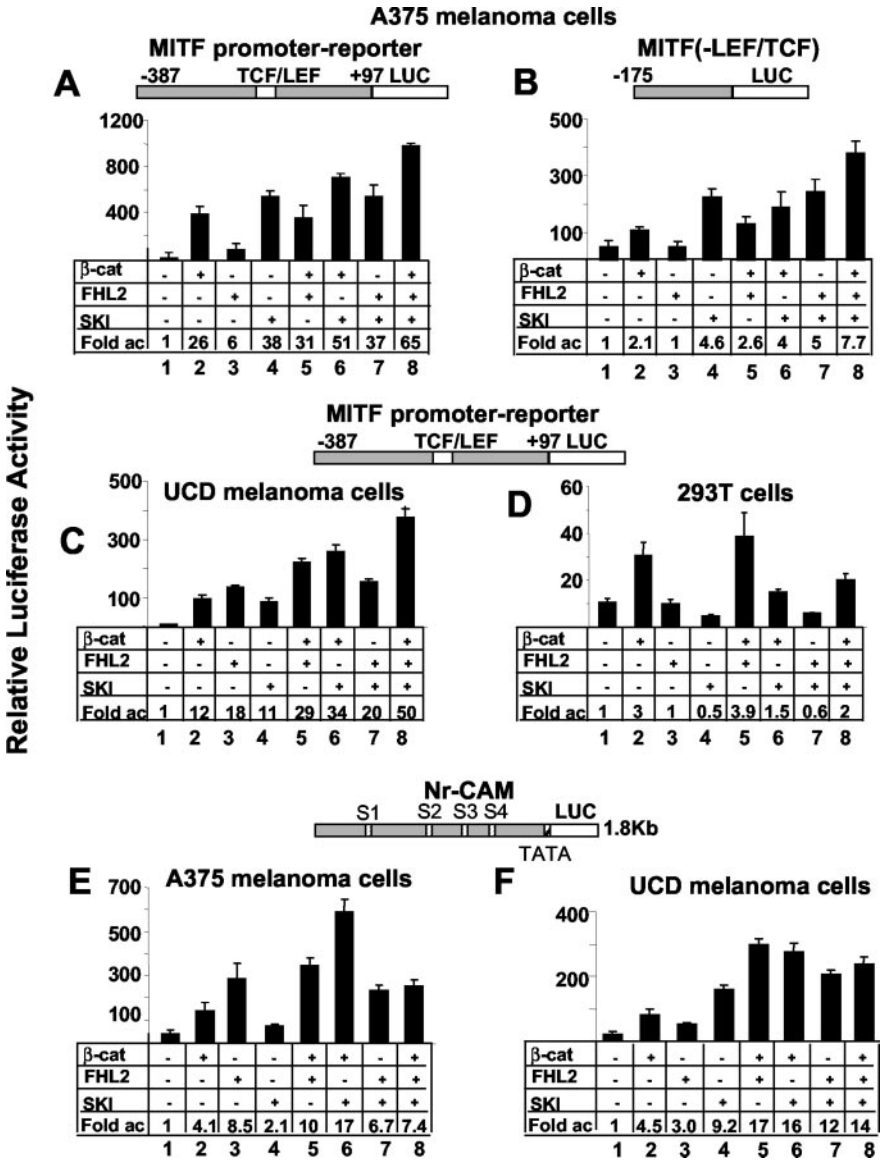

Fig. 4. SKI activates the MITF and Nr-CAM promoters. A375 and UCD melanoma cells were transfected with MITF promoter-reporters or Nr-CAM reporters in the presence or absence of combinations of SKI, FHL2, and β-catenin. *A* and *C*, SKI is a potent activator of the MITF promoter in the human melanoma cells A375 and UCD. *B*, deletion of the TCF/LEF binding site in the MITF promoter restricts the activation capacity of SKI in A375 melanoma cells. *D*, β-catenin and FHL2 but not SKI activate the MITF promoter in 293T cells. *E*, SKI and β-catenin synergistically activate the Nr-CAM promoter in A375 cells. *F*, SKI by itself is a stronger activator of the Nr-CAM promoter than either β-catenin or FHL2 in UCD cells.

levels of SKI appear to repress the apoptotic activity of FHL2, becoming a growth stimulator.

Next, we studied in more detail some properties resulting from the overexpression of SKI in the stable melanoma cell line (UCD(SKI$^+$), including cell cycle distribution, clonogenicity, and migration. As it was previously observed in transiently transfected cells (Fig. 4), stable overexpression of SKI significantly increased MITF and Nr-CAM mRNA levels compared with cells expressing the empty vector (Fig. 5*C*). In agreement with it growth-stimulatory activity (Fig. 5*B*), overexpression of SKI reduced the number of cells in the G_1 phase of the cell cycle and increased the number of cells in S phase (Fig. 5*D*). In addition, overexpression of SKI resulted in elevated clonogenic capacity and colony size (Fig. 5*E*, *top panels*). A comparison of colony phenotypes between melanoma cells transduced with pBabe control and pBabe-*SKI* vectors indicated that the large colonies induced by SKI were formed by a central core of tightly packed cells and loose cells in the periphery (Fig. 5*E*, *bottom panels*). This was indicative of increased cell motility in SKI overexpressing cells and demonstrated by the accelerated ability of wound closing compared with cells transfected with the empty vector (Fig. 5*F*). Taken together, these results showed that SKI is a powerful regulator of the growth, clonogenic ability, and motility of cells from melanocytic origin.

DISCUSSION

SKI Regulates β-Catenin Signaling. We have identified FHL2 as a SKI-interacting protein. SKI and FHL2 coimmunoprecipitated from cell extracts and showed a strong interaction in a yeast two-hybrid assay. Although yeast two-hybrid interactions of mammalian proteins are often direct, the possibility remains that the SKI-FHL2 interaction was mediated by a yeast protein. Thus, we propose that SKI and FHL2 interact directly but do not rule out the possibility that the interaction is indirect and mediated by a third as yet unidentified protein. Aberrant activation of β-catenin signaling by either mutations in β-catenin (52) or by the elevation in wild-type β-catenin nuclear content (53) has been linked to melanoma progression. We demonstrated that SKI and FHL2 activated β-catenin signaling in human melanoma cells. Importantly, the FHL2-interacting domain resides within amino acids 99–274 of the SKI molecule. This same domain is required for its association with N-CoR and the transcriptional repression activities of SKI (54). This suggests that distinct SKI complexes, having repressive or activating activities, may coexist in the cell and, by targeting different promoters, diversify the functions of SKI. Alternatively, chromatin-remodeling proteins may switch SKI complexes from repressors to activators. The repressive activity of SKI/Smad complexes

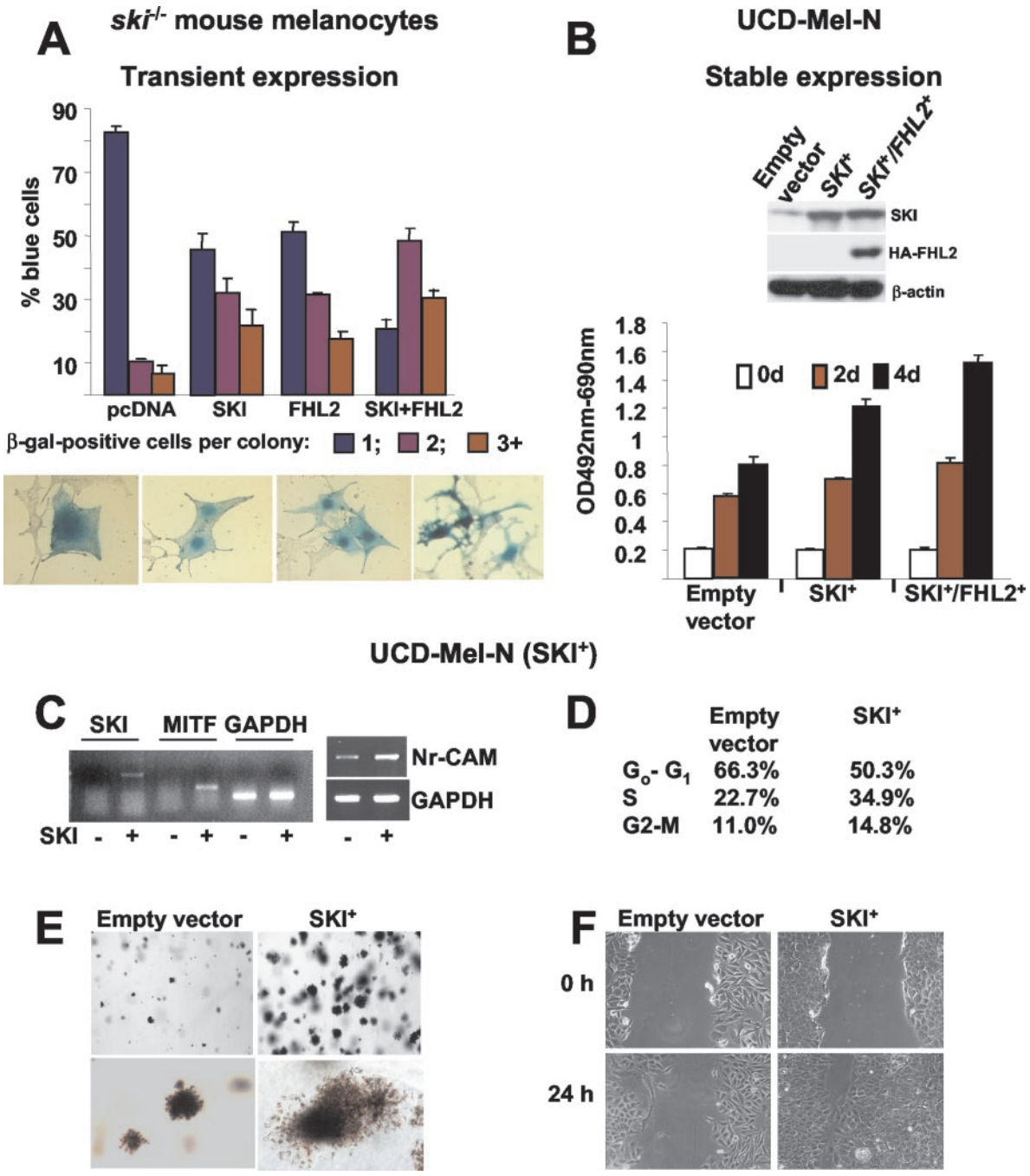

Fig. 5. SKI stimulates melanocyte and melanoma proliferation, cell cycle alterations, and clonogenicity. *A*, $ski^{-/-}$ melanocytes were transiently cotransfected with SKI, FHL2, or SKI and FHL2 and a β-gal expression plasmid as indicated in "Materials and Methods." At day 3, the melanocytes were fixed and stained for β-gal activity. The percentage of blue colonies is presented. The majority of cells transfected cells with SKI and FHL2 plasmids contained two or more cell clusters indicating rapid cell division compared with empty vector-transfected cells. The data are the average of triplicate measurements ± the SE. A typical picture of blue cells is shown below for day 3 after transfection. *B*, proliferation of UCD melanoma cells and derivatives overexpressing SKI (UCD(SKI^+) and SKI plus FHL2 (UCD(SKI^+/FHL^+) was assessed using the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt assay ("Materials and Methods"; *bottom panel*). The *top panel* indicates expression levels of SKI and HA-FHL2. *C*, RT-PCR analysis of mRNA isolated from UCD-Mel-N cells stably overexpressing SKI (UCD(SKI^+) shows high levels of MITF and Nr-CAM compared with the parental cell line expressing empty vectors. *D*, cell cycle distribution profiles show that UCD(SKI^+) cells display an increased number of cells in the S phase of the cell cycle and a decrease in G1 cells. *E*, UCD(SKI^+) were seeded in 6-well dishes containing 0.33% low-melt agarose as described previously (5). *Top* and *bottom panels* represent pictures taken with ×10 and ×40 objectives, respectively. *F*, an artificial wound was introduced with a micropipette tip into confluent cultures of pBabe and pBabe-ski cells; wound closure was followed for 24 h. Pictures were taken with ×10 objectives.

is associated with N-CoR, mSin3 and the histone deacetylase HDAC1 (11, 54). We speculate that a competition between histone acetylases and deacetylases for SKI complexes may regulate its transcriptional activities because addition of the histone acetyltransferase p300/CBP (Fig. 3) or the histone deacetylase inhibitor trichostatin A (data not shown) additionally enhanced SKI activity on β-catenin-responsive gene promoters in transient transfection experiments.

SKI Is an Activator of the MITF and Nr-CAM Genes. MITF is a major regulator of melanocyte development and survival (47). Recent evidence indicates that β-catenin is a potent mediator of melanoma growth by mechanisms involving MITF (48). MITF can target the expression of Bcl2, and disruption of MITF in melanoma cells induces massive apoptosis (48). In addition, MITF cooperates with LEF-1, a key transducer of the Wnt signal, to transactivate the

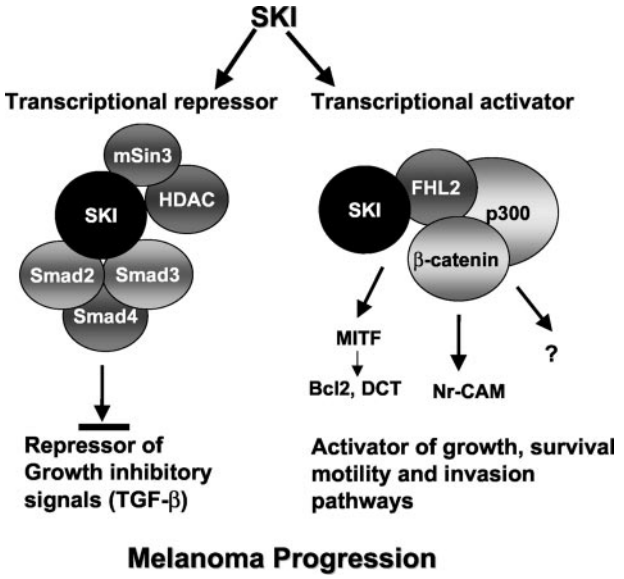

Fig. 6. Overexpression of SKI results in insensitivity to growth inhibitory signals and stimulation of growth, antiapoptotic and invasion pathways. *Bcl*II (48) and DCT (55) are MITF downstream targets. For details, see text.

DCT gene promoter (47, 55). The DCT protein is an early melanoblast marker and a mediator of the melanoma-specific resistance to *cis*-diaminedichloroplatinum (47, 56). Thus, activation of the β-catenin pathway and MITF expression appears to be essential for growth and survival of melanoma cells. Our data indicates that SKI is a more potent MITF activator compared with β-catenin (Fig. 4*A*, *Lanes 2* and *4*).

It is important to mention that MITF expression is insufficient to stimulate cell cycle progression. Therefore, other downstream targets of β-catenin might be responsible for the induction of cell proliferation (37). One such candidate appears to be Nr-CAM because its expression is elevated in human melanoma cells, and its overexpression stimulates cell proliferation, motility, and tumorigenicity (23). In human melanoma cells, SKI and β-catenin were more potent activators of the Nr-CAM promoter than FHL2 and β-catenin (Fig. 4), whereas stable overexpression of SKI was sufficient for increasing Nr-CAM mRNA over the basal levels (Fig. 5). The variability of SKI activity on MITF and Nr-CAM promoters suggests that the stoichiometry of SKI-protein complexes may be promoter specific and relevant for SKI function.

SKI Is a Key Regulator of Melanoma Progression. Inactivation of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ is associated with familial melanomas (reviewed in Ref. 57). However, molecular defects in the p16 locus have not been detected in the majority of human melanomas (58). Recently, BRAF mutations were found in 66% of human melanomas (59). However, BRAF alone is insufficient for the development of malignant melanoma because 82% of histologically diverse nevi, including congenital, intradermal, compound, and dysplastic, also display BRAF mutations (60). Thus, in addition to BRAF mutations and activation of the RAS/MAPK pathway, other molecular lesions are apparently required for the progression to invasive melanoma.

Overexpression of SKI correlates with melanoma invasion and progression *in vivo* (5), and recent studies suggest that high levels of SKI in association with Skip inactivates the RB pathway in a manner similar to $p16^{INK4a}$ loss (15). In this study, we provided evidence that SKI and FHL2 enhance $ski^{-/-}$ mouse embryonic melanocyte growth, whereas SKI alone appears to be sufficient for accelerating human melanoma cell cycle transit and proliferation and for increasing clonogenicity, colony size, and motility. Furthermore, overexpression of SKI appears to change the cellular activities of FHL2, transforming it from an apoptosis inducer (Ref. 51 and data not shown) to a growth promoter (Fig. 5*B*).

In conclusion, by activating β-catenin signaling and repressing the TGF-β pathway, SKI becomes a key regulator of melanoma progression (Fig. 6). We propose that overexpression of SKI may trigger a cascade of events leading to increased cell cycle alterations and growth potential, invasion, and cell survival. Such a model predicts that SKI may be a valuable target in the treatment of human malignant melanoma.

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